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[54] Title of the Invention

Antifungal Agent with Peptide as Active Component

[57] Abstract

Objective To provide an antifungal agent useful in the fields of foodstuffs, medicine, construction materials and coatings, agriculture and horticulture and as feed for livestock and fisheries, and that moreover has excellent antifungal action

Constitution An antifungal agent possessing the peptides (a) or (b) below as the active ingredient thereof

(a) A peptide consisting of the following amino acid sequence:

Ala Lys Ile Pro Ile Lys Ala Ile Leu Thr Val Gly Leu Ala Val Gly Lys
Gly Leu Arg Ala Ile Asn Ile Ala Ser Thr Ala Asn Asp Val Phe Asn Phe
Leu Lys Pro Lys Lys Arg Lys His

(b) A peptide consisting of an amino acid sequence consisting of the above amino acid sequence in which one or a plurality of amino acids has been added, subtracted or substituted, and which possesses antifungal activity

Claims

Claim 1 An antifungal agent possessing the peptides (a) or (b) below as the active ingredient thereof

(a) A peptide consisting of the amino acid sequence described in Sequence No. 1

(b) A peptide consisting of an amino acid sequence consisting of amino acid sequence (a) in which one or a plurality of amino acids has been added, subtracted or substituted, and which possesses antifungal activity

Detailed description of the invention

Relevant area of industry

This invention relates to an antifungal agent whose active ingredient is a peptide derived from insects.

Prior art

The invasion of the body cavities of insects by bacteria prompts the production in the humours of the insects of proteins or peptides that possess bactericidal actions or propagation preventive actions against the bacteria. Many bactericidal proteins and bactericidal peptides have hitherto been isolated from various insects, and detailed investigations have been made of their antibacterial actions against bacteria.

However, insects also appear to eliminate fungi in the same manner as they eliminate bacteria. Fungi include moulds. The reduction of damage from moulds is an important issue in all fields of industry, and particularly in the fields of food hygiene and medicine. However, there has been virtually no investigation of the antifungal effects on fungi of these antifungal proteins and antifungal peptides.

The inventors of the present invention have discovered in the humours of silkworm larvae an antibacterial peptide that exhibits strong antibacterial actions against various Gram-positive and Gram-negative bacteria, which has been named moricin (JP8-119995; *J. Biol. Chem.* vol. 270, page 29923 (1995)). However, the antifungal effects of moricin against fungi were completely unknown.

Problem addressed by the invention

It is an objective of the present invention to provide an antifungal agent whose active ingredient is moricin.

Means employed in order to resolve the problem

The inventors of the present invention having discovered that moricin possesses an excellent anti-propagation effect against fungi were able to perfect

the present invention. Thus, the present invention relates to an antifungal agent possessing the peptides (a) or (b) below as the active ingredient thereof
(a) A peptide consisting of the amino acid sequence described in Sequence No. 1
(b) A peptide consisting of an amino acid sequence consisting of amino acid sequence (a) in which one or a plurality of amino acids has been added, subtracted or substituted, and which possesses antifungal activity. Such peptides (a) and (b) have the meaning of the aforementioned peptide moricin. Moreover, the antifungal agents envisaged by the present invention have the meaning of pharmaceuticals that possess bactericidal action or alternatively propagation inhibiting action against fungi. In this invention, bactericidal action or propagation inhibiting action against fungi are referred to as 'antifungal action'.

Mode of implementation of the invention

The following is a more detailed description of the present invention. Moricin is isolated by the normal methods of refining peptides from the humours of silkworm larvae in whom antimicrobial activity has been induced. The amino acid sequence of moricin derived from silkworm larvae (hereinafter referred to as 'Noda type moricin') is as described by Sequence Number 1. Noda type moricin can be isolated from the humours of silkworm larvae by the method revealed in JP8-119995. In this case, first *Escherichia coli* are injected into the body cavities of silkworm larvae to induce the production of moricin. Next, the humours of the silkworm larvae are collected and are salted out with ammonium sulphate. Next, the moricin is isolated by successively supplying the salted out product to a gel filtration column, a positive ion exchange column and reverse phase HPLC.

Noda type moricin may also be prepared from recombinant microorganisms and so forth by genetic engineering procedures. In this case, first the Noda type moricin genes are cloned from silkworm larvae, or alternatively genes that encode for the amino acid sequence of Noda type moricin are artificially synthesized. Next, the genes are introduced into an appropriate vector – host line to create a recombinant microorganism. Such recombinant microorganisms are propagated in an appropriate culture and the moricin may be recovered from the resulting growth. (*E. coli*) JM109 (pXAMOR1) (FERM-BP-5099) revealed by JP8-119995 may be employed as the recombinant microorganism.

The moricin envisaged by the present invention consists of the amino acid sequence in Sequence Number 1 in which one or a plurality of amino acids have been added, subtracted or substituted, and may also be a peptide that possesses antifungal activity (hereinafter referred to as 'variant moricin'). When variant moricin is prepared, first variant moricin genes are constructed in which addition, subtraction or substitution variants have been introduced into the base sequence of Noda type moricin genes. Next, the variant gene is introduced into an appropriate vector – host to form the recombinant micro-

organism. The substance that produces the peptide that possesses antifungal action, that is, the variant moricin, is screened from the recombinant microorganism. The microorganism that is capable of producing the variant moricin is propagated in an appropriate culture, and the variant moricin may be harvested from the product.

The introduction of the variation to the Noda type moricin genes may be through contact between the genes and a mutagenic chemical substance, and more specifically, hydroxylamine, nitrous acid, sulphurous acid, and 5-bromouracil and so forth. Moreover, the variant moricin genes may also be constructed that possess the variation in the desired position by annealing chemically synthesized oligonucleotides. Apart from this, the ultraviolet radiation method, cassette mutagenesis method and PCR method to introduce a location-specific mutation, and the introduction of random mutation are also widely employed.

The moricin employed in the present invention is prepared by the chemical synthesis of the peptide. One or a plurality of the amino acids in the amino acid sequence of the moricin envisaged by the present invention may also be modified. Such modification of the amino acids may be the addition of for example phosphates or saccharide chains, or by acylation, alkylation, amidation or hydroxylation and so forth. The active ingredient of the antifungal agent envisaged by the present invention is moricin and possesses antimicrobial action or propagation inhibiting action against fungi. The fungi may be yeasts, mushrooms and toadstools and so forth and moulds, and more specifically may be vegetable pathogen moulds such as *Cucurbitaceae* vine split pathogenic fungus and tomato plague pathogenic fungus and so forth.

The antifungal agents envisaged by the present invention may be employed in a broad range of areas such as in foodstuffs, medicine, building materials and coatings, agriculture and horticulture and feed for livestock and fisheries and so forth. Moricin may be employed alone as the antifungal agent envisaged by the present invention. Moreover, the antifungal agent may be prepared by mixing with a solid carrier, liquid carrier or emulsified dispersion and may then be prepared in the form of tablets, powders, solutions, emulsions or capsules and so forth. Such carriers may be water, alcohol, gelatine, starch, magnesium stearate, lactose, mineral powders, gum Arabic, or vegetable oils and so forth.

When moricin is added to foodstuffs as a mould preventive, the moricin may for example be mixed with the food or coated onto the surface of the food and so forth. In such cases, not less than 2 mg per 1 kg, and preferably from 4 mg to 40 mg per 1 kg, should be added. No particular restrictions apply to the types of foodstuffs which may be employed, but seafood preparations such as

kamaboko and *chikuwa*[1] and so forth, processed meat products such as ham and sausage and so forth, beverages such as carbonated beverages and fruit beverages and so forth and confectionery such as cakes, custard puddings and *manju*[2] and so forth may be envisaged.

When moricin is employed as an agent to prevent rot in agriculture and horticulture, it may be mixed with the soil or with fertilizer, added to water for hydroponic culture, or scattered or painted onto plant seedlings or shoots or onto the soil and so forth. When moricin is mixed with soil or fertilizer, normally not less than 5 mg per 1 kg of soil or fertilizer, and preferably from 10 mg to 100 mg per 1 kg of soil or fertilizer, is employed.

Solutions of moricin may be prepared by mixing not less than 2 mg of moricin per 1 l of liquid carrier, and preferably from 4 mg to 40 mg per 1 l of liquid carrier. Water or alcohol and so forth may be employed as the liquid carrier. Antifungal agents, drugs, and food additives and so forth may be employed as appropriate together with moricin in the antifungal agents envisaged by the present invention.

Example of preparation of moricin (1) Isolation of moricin from silkworm larvae in which antifungal activity has been induced

① 700 silkworm larvae (variety name: Tokai x. Asahi, obtained from the Ministry of Agriculture, Forestry and Fisheries Silkworm and Insect Technical Laboratories) were injected in the body cavity with 0.005 ml per head of a suspension of *Escherichia coli* (HB101 ATCC33694) in physiological saline solution (4×10^8/ml) to induce antifungal activity. After 20 hours, the feet of the larvae were amputated and the humours were collected and were immediately heated for 5 minutes in a bath at 100° C and then were centrifugally separated.

② Ammonium sulphate was added to 15% saturation to 200 ml of the supernatant obtained from centrifuging and the material that was salted out was centrifugally separated. Ammonium sulphate was added to 75% saturation to the centrifuge supernatant and the material salted out was centrifugally separated and collected. The salted out matter was dissolved in 40 ml of distilled water.

③ The solution was passed through a Sephadex G-50 column (5 x 100 cm) buffered with 50 mM ammonium acetate (pH 5.0) and was gel filtered, and the low molecular weight protein fraction was collected.

④ The low molecular weight protein fraction was passed through a CM Sepharose FF column (2.5 x 4.4 cm) buffered with 50 mM ammonium acetate (pH 5.0). The fraction adsorbed was eluted by means of the passage through the column of successively mixtures in volume ratios of 9.5:0.5, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6 and 3:7 of 50mM ammonium acetate (pH 5.0) and 0.8 M ammonium acetate (pH 7.0). The eluate was taken 50 ml at a time

[1] Types of boiled fish paste - Translator
[2] Type of jam-filled dumpling - Translator

according to the salt concentration. The fraction eluted with the 4:6 mixture provided the strongest antimicrobial action.

⑤ 10 ml of the fraction eluted with the 4:6 mixture was supplied to a reverse phase HPLC (column: Capcell PaK C8 SG300 10 x 250 mm). The adsorbed fraction was eluted by concentration gradient with acetonitrile containing 0.1% trifluoroacetic acid. The remaining eluate was supplied to the reverse phase HPLC and the fraction exhibiting the strongest antimicrobial action was collected.

⑥ This fraction was again supplied to a reverse phase HPLC (column: Capcell PaK C8 SG300 4.6 x 250 mm) and further refined. The adsorbed fraction was eluted by concentration gradient with acetonitrile containing 0.1% trifluoroacetic acid. Portion of the eluate fraction was supplied to tricine-SDS polyacrylamide gel electrophoresis (hereinafter referred to as 'tricine-SDS PAGE'); after electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (manufactured by BRL) and a single stain band was obtained.

⑦ 150 μg of moricin was derived from 200 ml of the centrifugal supernatant from silkworm larvae by this method. The indicator for the measurement of antimicrobial action when moricin is isolated is circular formation inhibition on a flat plate (*Nature*, vol. 292, page 246 (1981)), and *Staphylococcus aureus* subsp. *aureus* ATCC 6538P was employed as the test microorganism. The measurement of protein was performed by the modified Laurie method (*Methods Enzymol.*, vol. 91, page 95 (1983)), and the tricine-SDS PAGE method described in *Anal. Biochem.*, vol. 166, page 368 (1987) was employed.

⑧ Structural analysis

(a) Amino acid component

The sample was subjected to hydrolysis (reduced pressure, 110° C, 24 hours) in 6N hydrochloric acid containing 0.1% phenol, and results of analysis by amino acid analyzer (manufactured by Hitachi, model 835) were as follows:

Amino acid	Mols/1 mol moricin
Lys	8.8
Ala	5.9
Ile	4.8
Asp + Asn	4.0
Gly	3.1
Val	3.2
Arg	1.7
Leu	2.1
Phe	2.3
Pro	1.9
Thr	1.9
His	1.4
Ser	1.0

(b) Molecular weight

Analysis by means of a molecular weight analyzer (manufactured by Perkin Elmer ScieX, model API-III) gave a result of 4543.1 ±0.6 Da.

(c) N-terminal amino acid sequence

The moricin N-terminal amino acid sequence was analyzed by the Edman method by protein sequencer (manufactured by Applied Biosystems, model 473A) and the amino acid sequence described in Sequence Number 2 was obtained.

(d) End proteinase digestion product

The moricin was digested in end proteinase Asp-N (manufactured by Takara Sake) and the peptide fragments produced were isolated by reverse phase HPLC. The amino acid sequences of each of the fragments were analyzed by Edman's method and the amino acid sequences described in Sequence Number 3 and Sequence Number 4 were obtained.

(e) As a result of the amino acid analysis, qualitative analysis and analysis by Edman's method, it was concluded that the moricin was a peptide possessing the amino acid sequence described in Sequence Number 1.

Example of preparation of moricin (2) Production of moricin by genetic engineering method

① Construction by chemical synthesis of DNA containing moricin genes[3]

(a) Determination of base sequence

The codon frequently used with *Escherichia coli* was employed (Ikemura, T., *J. Mol. Biol.*, vol. 151, pages 389 – 409) (1987)) and the base sequence was determined for the DNA to be constructed, that is to say, DNA containing moricin genes. The structure of the DNA was the codon for methionine for exclusion by cyanogen bromide (ATG), moricin gene, two terminal codons (TAA and TAG) below the EcoRI restriction enzyme recognition site, followed by the Sa11 recognition site. The base sequence that was determined for the moricin gene is shown at Sequence Number 5.

(b) Chemical synthesis of oligonucleotide

The DNA containing the moricin gene was divided into eight parts (mosy1 to mosy8) and chemically synthesized. A DNA synthesizer (manufactured by Applied Biosystems, ABI model 392) was employed for the synthesis of the oligonucleotides and was performed by the phosphoamidite method. The base sequences of mosy1 to mosy8 were as follows:

mosy1 AATTCATGGCTAAAATCCCGATTAAGGCAATTAAA
mosy2 ACTGTGGGCAAAGCTGTTGGTAAAGGTCTGGGTG
mosy3 CTATCAACATGGCTTCTACCGCTAACGAAGTATTCAA
mosy4 CTTCCTGAAACCGAAGAAACGTAAACACTAATAG
mosy5 CACAGTTTTAATTGCCTTAATCGGGATTTTAGCCATG
mosy6 GTTGATAGCAGCCAGAACTTTACCAACAGCTTTGCC
mosy7 CAGGAAGTTGAATACGTCGTTAGCGGTAGAAGCGAT
mosy8 TCGACTATTAGTGTTTACGTTTCTTCGGTTT

[3] The numbering in the Japanese original becomes irregular from this point. I have retained the original numbering. - Translator

(c) Refining of synthesized oligonucleotides

A QIYAGEN Plasmid Kit (manufactured by Funakoshi) was employed for the refining of the synthesized oligonucleotides. First, a portion of the oligonucleotides synthesized under (b) was dissolved in MOPS solution (50 mM MOPS (pH 7.0) and 0.1 M NaCl) and a 20 μg/ml oligonucleotide solution was prepared. 1 ml of this oligonucleotide solution was supplied to a refining tube (QIYAGEN-tip 20) buffered with 2 ml of a buffer solution (50 mM MOPS (pH 7.0), 0.1 M NaCl and 0.15% Triton X-100), and after the oligonucleotides had been adsorbed, it was rinsed with 4 ml of MOPS solution. Next the oligonucleotides were eluted, precipitated and rinsed according to the manual provided with the kit. The oligonucleotides derived (6 μg to 13 μg) were dissolved in 15 μl of TE solution (1- mM Tris-HCl (pH 8.0) and 1 mM EDTA).

(d) Phosphorylation of oligonucleotides

The 5′ terminals of the oligonucleotides of mosy2 to mosy8 were phosphorylated by means of ATP and T4 polynucleotide kinase. The reactions were conducted in separate tubes and the conditions were as follows. 3 μg of oligonucleotide (dissolved in TE solution), 2 μl of 10x kination buffer (500 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine-HCl and 1 mM EDTA, manufactured by Nippon Gene), 2 μl of 10 mM ATP and 20 U of T4 polynucleotide kinase (manufactured by Nippon Gene) were mixed together, water was added to a total of 20 μl and the mixture was reacted for 2 hours at 37° C. The mixture was then heated for 3 hours at 90° C and the reaction was stopped.

(e) Construction of DNA including moricin genes

(i) Annealing of oligonucleotides

120 μl in aggregate of the phosphorylated reaction solutions of mosy2 to mosy7 was placed in a single tube to which 3 μg of mosy1 and 3 μg of mosy8 were added. 20 μl of ligation buffer (500 mM Tris-HCl (pH 7.9), 100 mM $MgCl_2$, 200 mM DTT and 10 mM ATP, manufactured by Nippon Gene) was also added, whereupon water was added to a total of 197 μl. The mixture was heated for 5 minutes at 90° C and immediately chilled and was then heated for 10 minutes at 75° C by heat block (Dry Thermo Unit model AL-10, manufactured by Taitech). The power supply to the heat block was turned off and the mixture was allowed to cool for 5 minutes.

(ii) Ligation of oligonucleotides

1800 U of DNA ligase (manufactured by Nippon Gene) was added to 197 μl of the reaction solution, and the mixture was reacted for 14 hours at 16° C.

(iii) Refining of DNA

3 μl of BPB solution (0.1% BPB and 30% glycerol) was added to portion (30 μl) of the reaction solution, 3% agarose gel electrophoresis was performed and the approximately 140 bp chain

length band was isolated. The DNA in the gel was recovered by freezing and thawing and ethanol precipitation. 800 ng of the resulting DNA was dissolved in 9 μl of 1x kination buffer.

(iv) Phosphorylation of DNA containing moricin gene

1 μl of 10 mM ATP and 10 U of T4 polynucleotide kinase was added to 8 μl of the above DNA solution and the mixture was reacted for 2 hours at 37° C. The solution was then heated at 90° C for 3 minutes and the reaction was stopped.

(b) Preparation of recombinant DNA (pUCMOR1 plasmid)

The DNA containing the moricin gene was inserted into the pUCMOR1 plasmid. The plasmid was obtained in the following manner. 4 μg of plasmid pUC119 (manufactured by Takara Sake), 4 μl of 10xH buffer (500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 10 mM DTT and 1000 mM NaCl, manufactured by Takara Sake), 20 U of EcoRI restriction enzyme (manufactured by GIBCO BRL) and 20 U of SalI (manufactured by Takara Sake) were mixed together and water was added to a total of 40 μl, whereupon the mixture was digested for 20 hours at 37° C. The mixture was then heated for 15 minutes at 65° C and the reaction was stopped.

2 μl of the reaction solution (equivalent to 200 ng of plasmid), 10 μl of the DNA containing the moricin gene as prepared at 1. (5) above, 2 μl of 10x ligation buffer, 2 μl of 2 μg/ml BSA and 1200 U of T4 DNA ligase were mixed together and water was added to a total of 20 μl, whereupon the mixture was ligation reacted for 6 hours at 16° C. 20 μl of the ligation reaction solution was mixed with 100 μl of competent cell (*Escherichia coli* JM109, manufactured by Nippon Gene) solution and was then held for 30 minutes on ice, for 2 hours at 37° C and for 5 minutes on ice and transformed. Next, 400 μl of high competent broth (manufactured by Nippon Gene) was added and shaking cultured for 1 hour at 37° C, and the mixture was divided into four equal parts and spread over four 2XYT (1.6% Trypton, 1% yeast extract and 0.5% NaCl) agar-agar cultures containing 50 μg/ml of ampicillin, 0.1 mM IPTG and 40 μg/ml X-gal, cultured for 20 minutes at 37° C, which produced approximately 300 ampicillin-resistant colonies.

Twenty pale blue ampicillin-resistant colonies were selected and were cultured in 3 ml of LB liquid culture containing 50 μg/ml of ampicillin for 10 hours at 37° C. The culture solution was centrifuged and the microorganisms were concentrated, whereupon the plasmid DNA was refined by means of a QIYAGEN Plasmid Mini Kit (manufactured by Funakoshi) and the product was dissolved in 20 μl of TE solution. 2 μl of this plasmid solution, 1 μl of 10xH buffer, 3 U of EcoRI restriction enzyme and 3 U of Sal1 restriction enzyme were mixed together and water was added to total 10 μl whereupon the solution was reacted for 90 minutes at 37° C. 1 μl of BPB solution was added to the reaction solution and 4% agarose electrophoresis was performed, and the chain length of the insert DNA isolated

by the restriction enzymes was confirmed. Various large fragments of 120 bp to 140 bp were recognized. The base sequences for the 14 plasmids possessing 140 bp insert DNA were determined. The base sequences were determined by employing a Tag Dye Primer Cycle Sequencing Kit (manufactured by ABI), a GeneAmp PCR System 9600 (manufactured by Perkin Elmer Cetus) and a DNA sequencer (model 373A, manufactured by ABI). Four plasmids contained DNA including moricin genes, and one of these was named pUCMOR1.

(c) Preparation of moricin-expressing recombinant DNA (plasmid pXAMOR1)

Plasmid pXAMOR1 was derived by subcloning the DNA containing the moricin gene inserted in plasmid pUCMOR1 in the EcoR and Sal1 locations in plasmid pXa1. This plasmid was restricted by lac promoter/operator and expressed fused protein consisting of β-galactosidase, collagen fragments and moricin. This plasmid was derived in the following manner. 5 μg of plasmid pXa1 (manufactured by Behringer), 10 μl of 10xH buffer, 20 U of EcoRI restriction enzyme and 20 U of Sal1 restriction enzyme were mixed together and water was added to a total of 100 μl, whereupon the mixture was digested for 20 hours at 37° C. The mixture was then heated for 15 minutes at 65° C and the reaction was stopped, and fragmentary pXa1 plasmid was obtained. Plasmid pUCMOR1 was digested with EcoRI and Sal1 and the insert DNA was isolated, whereupon agarose electrophoresis was employed in order to refine the insert DNA. The conditions were as follows.

10 μg of pUCMOR1 plasmid, 5 μl of 10xH buffer, 20 U of EcoRI restriction enzyme and 20 U of Sal1 restriction enzyme were mixed together and water was added to a total of 50 μl, and the mixture was then reacted for 20 hours at 37° C. 3 μl of BPB solution was added to 50 μl of the reaction solution, 3% agarose electrophoresis was performed, and the approximately 140 bp chain length band was isolated. The DNA in the gel was then recovered by freezing and thawing and ethanol precipitation. 60 ng of the insert DNA so derived was dissolved in 18 μl of water. 20 ng of the refined insert DNA, 1 μl (50 ng) of the fragmentary pXa1 plasmid, 1 μl of 10x ligation buffer, 1 μl of 2 μg/l BSA and 600 U of T4 DNA ligase were then mixed together and water was added to a total of 10 μl, and the mixture was ligation reacted for 5 hours at 16° C.

This ligation solution was mixed with 100 μl of competent cells (*Escherichia coli* JM109, manufactured by Nippon Gene), and was held on ice for 30 minutes, at 37° C for 2 minutes, and on ice for 5 minutes for transformation. The reaction solution was spread onto two 2xYT agar-agar cultures containing 50 μg/ml of ampicillin and cultured for 20 hours at 37° C to yield approximately 1000 ampicillin-resistant colonies. 14 of these ampicillin-resistant colonies were selected and were cultured in 3 ml of LB solution containing 50 μg/ml of ampicillin for 10 minutes at 37° C. The

culture liquid was centrifuged to concentrate the microorganisms and the plasmids were then refined by means of a QIYAGEN Plasmid Mini Kit and were dissolved in 20 μl of TE solution.

2 μl of this plasmid solution, 1 μl of 10xH buffer, 3 U of EcoRI restriction enzyme and 3 U of Sal1 restriction enzyme were mixed together and water was added to a total of 10 μl, 1 μl of BP solution was added to the reaction solution, 4% agarose electrophoresis was performed and the chain length of the insert DNA isolated by the restriction enzymes was confirmed, with all plasmids being confirmed to contain insert DNA with 140 bp chain lengths. One of these plasmids was named pXAMOR1. The recombinant *Escherichia coli* containing pXAMOR1 was named (*E.* coli) JM109 (pXAMOR1) and was deposited as FERM BP-5099 at the Bioengineering Industrial Technology Laboratories of the Industrial Technology Institute.

(d) Production of moricin-fused protein by means of recombinant microorganism

(i) Confirmation of production of fused protein

One colony of recombinant *Escherichia coli* JM109 (pXAMORI1) containing the pXAMOR1 plasmid was inoculated into 2 ml of LB solution containing 50 μg/ml of ampicillin and was cultured for 2 hours at 37° C. 10 μl of 100 mM IPTG was then added and the expression of the fused protein was induced and the culture was again cultured for 2 hours at 37° C. 0.5 ml of the culture solution was centrifuged to concentrate the microorganisms and then the bacteria were suspended in 100 μl of sample buffer (62.5 mM Tris-HCl (pH6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.0025% BPB) and heated for 5 minutes at 100° C. 3 μl of this was supplied to SDS-PAGE. As a control, culture solution of recombinant *Escherichia coli* to which IPTG had not been added was also supplied to SDS-PAGE. The predicted molecular weight of β-galactosidase and collagen fragments and moricin was 127.5 kD. A band at approximately a molecular weight of 127.5 kD was confirmed only in the culture to which the IPTG was added, and it was confirmed that the fused protein was being expressed.

(ii) Partial refining of fused protein

The recombinant *Escherichia coli* was cultured 40 ml of 2xYT liquid culture medium containing ampicillin for 14 minutes at 37° C. Next, 10 ml of the aforementioned culture was placed in each of three flasks each containing 300 ml of 2xYT liquid culture medium containing 50 μg/ml of ampicillin and was cultured for 2 hours at 37° C. 1.5 ml of 100 mM IPTG was added to each of the flasks and the cultures were cultured for a further 2 hours, the cultures solutions were centrifuged in order to concentrate the bacteria, and 3.7 g of the bacteria was derived. The bacteria were suspended in 35 ml of a suspension solution (10 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA (pH 8.0) and 10 mM 2-mercaptoethanol). The suspension was subjected to freezing and thaw-

ing and ultrasound processing (Sonifier Cell Disruptor W200P, manufactured by Branson) and the bacteria were destroyed. The product was then centrifuged and the precipitate was suspended in 35 ml of a suspension solution (0.5% Triton X-100 and 10 mM EDTA (pH 8.0). This was again centrifuged and the supernatant was removed to yield 9 mg of protein. This became the partially refined protein which was then supplied to cyanogen bromide treatment.

(iii) Cyanogen bromide treatment

200 µl of 70% formic acid and 10 µl of 100 mg/ml of cyanogen bromide (dissolved in acetonitrile) was added to 1 mg of the partially refined protein derived by the method described in the foregoing and the mixture was reacted for 24 hours at room temperature, and then dried under reduced pressure. This formed the partially refined moricin.

Practical embodiments

The following is a more detailed description of the present invention by means of practical embodiments thereof. However, the technical scope of the present invention is not restricted thereto.

Practical Embodiment 1 Test of antifungal activity of moricin

In order to examine the antifungal activity of moricin in relation to vegetable pathogenic moulds, the minimum propagation inhibitory concentration (MIC) was determined by the method described in *FEMS Microbiol. Lett.*, vol. 69, page 55 (1990).

1. Substances for testing

(1) Moricin: Partially refined moricin derived by the recombinant gene procedure of the example of manufacture (2) described above was successively provided to gel filter chromatography employing Sephadex G-50 (manufactured by Pharmacia Fine Chemicals), cation exchange column chromatography employing CM Sepharose FF (manufactured by Pharmacia Fine Chemicals) and reverse phase HPLC employing Capcell Pak C8 SG300 (manufactured by Shiseido), and the moricin was refined until near purity. Next, the molecular weight of the refined moricin was measured by mass analyzer and the N terminal amino acid sequences were analyzed and the amino acid sequences of the end proteinase digest were analyzed, and it was confirmed that they were identical with the amino acid sequence shown in Sequence Number 1.

(2) Cecropin B2: Cecropin B2 which is an antibacterial peptide derived from silkworms (*Comp. Biochem. Physiol.*, vol. 95B, page 551 (1990)) was obtained as a comparison from the Silkworm and Insect Agricultural Technology Laboratories of the Ministry of Agriculture, Forestry and Fisheries.

(3) Amphotericin B: Amphotericin B (trade name *Fungicin*, manufactured by GIBCO BRL) was employed as a comparative antifungal agent.

2. Bacteria for investigation

(1) *Cucurbitaceae* vine split pathogenic fungus (*Fusarium oxysporum Schlechtendahl cucumerinum* IF06384)

(2) Tomato plague pathogenic fungus (*Phytophthora infestans*) (obtained from Nippon Del Monte)

3. Method of preparation of conidiospores or zoospores

(1) When the organism to be tested was *Cucurbitaceae* vine split pathogenic fungus, it was cultured at 30° C for 5 days on a potato dextrose (PD) agar-agar culture medium (manufactured by Difco). An appropriate amount of sterilizing solution was added to the culture and the conidiospores were suspended. Portion of the suspension was transferred to a blood cell count plate, and the number of conidiospores was counted under the microscope (standard model, manufactured by Olympus). The suspension was diluted with PD liquid culture medium (manufactured by Difco) in such a manner as to yield approximately 20,000 conidiospores per 1 ml.

(2) When the organism to be tested was the tomato plague pathogenic fungus, it was cultured for 14 days at 20° C on PD agar-agar culture medium. An appropriate amount of sterilizing solution was added to the culture and the zoosporangia were suspended; the suspension was allowed to stand for 2 hours on ice in order to release the zoospores. 50 µl of the suspension was shifted to a 96 well micro-titre plate (flat-based, manufactured by Falcon), and the number of zoosporangia was counted under the microscope. The suspension was diluted with PD liquid culture medium in such a manner as to yield approximately 20 zoosporangia in the field of vision under 100x magnification.

4. Method of testing antifungal activity

The suspensions of conidiospores and zoospores prepared as above were injected at a rate of 50 µl per well into a 96 well micro-titre plate. After the 50 µl of aqueous solutions of each concentration of test substances had been added to each of the wells, they were static cultured for 48 hours at 28° C. However, the tomato plague pathogen was static cultured for 96 hours at 20° C. After culture, the extent of propagation of the hyphae was observed with the naked eye. The minimum concentration of the test substance at which absolutely no propagation of the test organisms was detected was deemed to be the MIC. The results are listed in Table 1.

Table 1

Test organism	MIC (µg/ml)		
	Moricin	Cecropin B2	Amphotericin B
Cucurbitaceae vine split pathogen	2.3	62.4	2.0
Tomato plague pathogen	1.1	7.8	15.6

As will be evident from the results in Table 1, the antifungal activity of moricin against the test organisms was equal to or greater than that of the known antifungal agent Amphotericin B. While Cecropin B2 also exhibited antifungal activity, it did not exhibit the level of activity exhibited by moricin. Because moricin is able to severely impede the propagation of vegetable pathogens, moricin is clearly of advantage as an antifungal agent.

Example of preparation 1: Moricin prepared by the method described under (1) of Practical Embodiment 1 was freeze dried and then dissolved in water to prepare a 100 mg/ml moricin solution, forming an aqueous mould preventive agent for agricultural and horticultural use.

Example of preparation 2: 100 mg of moricin prepared by the method described under (1) of Practical Embodiment 1, 40 g of clay and 60 g of talc were ground and mixed together to form a powder.

Example of preparation 3: 100 mg of moricin prepared by the method described under (1) of Practical Embodiment 1, 40 g of bentonite, 48 g of clay and 7 g of lignin sulphonic acid were uniformly mixed and water was added and the mixture was kneaded and then granulated and dried to form a granular preparation.

Effects of the invention

Moricin is provided as a novel antifungal agent that exhibits excellent antifungal action. The antifungal agents envisaged by the present invention may be employed over a broad range of applications such as foodstuffs, medicine, construction materials and coatings, agriculture and horticulture and livestock and fisheries feed and so forth.

Lists of sequences

Sequence Number: 1
Sequence length: 42
Sequence type: Amino acid
Topology: Straight chain
Sequence type: Peptide
Sequence

Ala Lys Ile Pro Ile Lys Ala Ile Lys Thr Val Gly Lys Ala Val
5 10 15
Gly Lys Gly Leu Arg Ala Ile Asn Ile Ala Ser Thr Ala Asn Asp
20 25 30
Val Phe Asn Phe Leu Lys Pro Lys Lys Arg Lys His
35 40

Sequence Number: 2
Sequence length: 33
Sequence type: Amino acid
Topology: Straight chain
Sequence type: Peptide
Sequence

```
Ala Lys Ile Pro Ile Lys Ala Ile Lys Thr Val Gly Lys Ala Val Gly
 1               5                  10                  15
Lys Gly Leu Arg Ala Ile Asn Ile Ala Ser Thr Ala Asn Asp Val Phe
            20                  25                  30
Asn
```

Sequence Number: 3
Sequence length: 29
Sequence type: Amino acid
Topology: Straight chain
Sequence type: Peptide
Sequence

```
Ala Lys Ile Pro Ile Lys Ala Ile Lys Thr Val Gly Lys Ala Val Gly
 1               5                  10                  15
Lys Gly Leu Arg Ala Ile Asn Ile Ala Ser Thr Ala Asn
            20                  25
```

Sequence Number: 4
Sequence length: 13
Sequence type: Amino acid
Topology: Straight chain
Sequence type: Peptide
Sequence

```
Asp Val Phe Asn Phe Leu Lys Pro Lys Lys Arg Lys His
 1               5                  10
```

Sequence Number: 5
Sequence length: 126
Sequence type: Nucleic acid
Topology: Two chain
Sequence type: Other nucleic acid Synthetic DNA
Sequence

```
GCT AAA ATC CCG ATT AAG GCA ATT AAA
ACT GTG GGC AAA GCT GTT GGT     48
Ala Lys Ile Pro Ile Lys Ala Ile Lys
Thr Val Gly Lys Ala Val Gly
            5                  10                  15
AAA GGT CTG CGT GCT ATC AAC ATC GCT TCT ACC GCT AAC GAC GTA TTC   96
Lys Gly Leu Arg Ala Ile Asn Ile Ala Ser Thr Ala Asn Asp Val Phe
            20                  25                  30
AAC TTC CTG AAA CCG AAG AAA CGT AAA CAC                      126
Asn Phe Leu Lys Pro Lys Lys Arg Lys His
        35                  40
```